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Breast Cancer is the most common non-skin malignancy in American women and is the second cause of cancer deaths in this population. Therefore, the need for new therapies is critical. These studies evaluated the therapeutic potential of a novel agent, the protein kinase C modulator, Bryostatin 1 in combination with the taxene, paclitaxel in breast cancer models, in vitro. The studies were designed to evaluate multiple treatment schedules of both agents to determine synergistic combinations. The combination of bryostatin 1 and paclitaxel was studied in four breast cancer cell lines utilizing multiple treatment schedules and no synergistic combination was identified. Several other chemotherapeutic agents (doxorubicin, cisplatin, 5-fluorouracil, & vinorelbine)were also tested in combination with bryostatin 1 using two breast cancer cell lines and three treatment schedules. Again, no synergistic combinations were identified. No synergistic combinations of bryostatin 1 and paclitaxel (as well as the other drugs tested) were identified even though several breast cancer cell lines and multiple treatment schedules were evaluated. These studies suggest that bryostatin 1 in combination with paclitaxel and the other drugs evaluated may not be a promising combination for the treatment of breast cancer.

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Table of Contents

The Effect of Protein Kinase C Modulation with Bryostatin 1 on Paclitaxel-Induced Growth Inhibition and Apoptosis in Human Breast Cancer

Hillary A. Hahm M.D., Ph.D.

Annual Report for Award DAMD17-97-1-7338

Introduction

Breast cancer is the most common non-skin cell malignancy in American women and is the second leading cause of cancer deaths in this group (1). Although there are many active cytotoxic chemotherapeutic agents currently available, the efficacy of these agents is limited by tumor cell resistance. Therefore the need for new therapies remains critical. One approach is to examine the addition of novel anti-tumor agents in combination with standard cytotoxic agents. This study utilized the novel agent, Bryostatin 1, in combination with the taxene, paclitaxel. Bryostatin 1 modulates Protein Kinase C (PKC) which is a critical enzyme in cell signal transduction (2). Bryostatin 1 has demonstrated direct anti-tumor activity as well as enhanced the anti-tumor effect of cytotoxic agents (3-6). Paclitaxel has demonstrated significant clinical activity against multiple tumor types including breast (7). The purpose of this study was to evaluate the hypothesis that PKC modulation by bryostatin 1 would augment paclitaxel-induced cytotoxicity in breast cancer cells. These pre-clinical studies were intended to form the foundation for the design of clinical studies in breast cancer patients utilizing combination therapy with paclitaxel and brystatin 1.

Body

Experimental Methods

The breast cancer cell lines, MCF 7, T47d, MDA MB 231, MDA MB 468, MDA MB 435 and Hs578t were utilized. These include both estrogen receptor positive and negative cell lines (8). Bryostatin 1 was obtained from the National Cancer Institute and maintained as a 1mM stock in DMSO (stored at -20^0C). Paclitaxel was a gift from Bristol-myers/Squibb. A concentrated paclitaxel solution 10mM in DMSO, was stored at -20^0C. Docetaxel was a gift from Rhone-Poulenc Rorer, a stock solution was made up in ethanol at 10mg/ml and stored at -20^0C. 5-Fluorouracil, vinorelbine, cisplatin were obtained from the oncology pharmacy. 5 Fluorouracil and cisplatin were stored at -20^0C and vinorelbine at 5^0C. Fluorodeoxyuridine (stock solution 10mM in water, stored at -20^0C) and doxorubicin (stock solution 10mM in DMSO, stored at -20^0C) were obtained from Sigma Co. Polyamine analogs N^1-[(cyclopropyl)methyl]-N^{11}-ethyl-4,8-diazaundecane (CPENSpm), and N^1-[(cycloheptyl)methyl]-N^{11}-ethyl-4,8-diazaundecane (CHENSpm) were obtained from the laboratory of Dr. Robert Casero (Johns Hopkins Oncology Center, Baltimore, MD). CPENSpm and CHENSpm were synthesized by Dr. Patrick Woster (Wayne State University, Detroit, MI). The polyamine analogs are dissolved in water for 10mM stock solutions, filter sterilized and stored at -20^0C. All drugs were diluted as required in cell culture medium then added individually to cell cultures using a range of concentrations from 1 X 10^{-10} M to 1 x 10^{-6} M (with vehicle treated cultures utilized as controls) to determine the growth

inhibition curves for each agent in each cell line. For combination studies, bryostatin 1 was utilized at three different concentrations, 1, 10 and 100 nM.

For growth inhibition studies, exponentially growing cells were plated in triplicate in 24 well or 96 well plates. Cell growth inhibition was determined by assessing % cell number or OD 540 in the treatment group versus control on day 5. Cells were detached with trypsinization and quantitation of cell number was done utilizing a coulter counter. Growth inhibition was also assessed utilizing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye assay. Prior to usage of the MTT assay to obtain experimental data, this assay was directly compared with cell counts and found to be consistent and comparable in measuring growth inhibition by drugs in the breast cancer cell lines. For the MTT assay, the cells were plated in 96 well plates and following completion of the culture period the media was discarded and 100 µl of MTT (5 mg/ml in culture medium, filter sterilized) was added to each well and the plates were incubated for 4 hours at 37^0C. The MTT solution was then removed and the formazan crystals were dissolved in 200 µl/well of a 1:1 (v/v) solution of DMSO:ethanol and color formation read at OD 540. Results were blanked against wells containing media but no cells for the culture period, and % growth inhibition was calculated by comparison of the treatment groups with the vehicle-treated control cells.

Combination studies were done to determine antagonism, additivity, or synergism. For combination studies where one agent has no growth inhibitory activity when added to the cell cultures, antagonism or synergy can be assessed by any significant change in the concentration of the second agent needed to produce the same level of growth inhibition (as seen with the second agent alone) when the first (inactive alone) agent is added. When both agents being utilized in combination studies were individually cytostatic or cytotoxic, the mathematical model for synergy of Chou and Talalay (9) was utilized. Based on this model, the cell cultures were treated with each drug individually at doses which would inhibit cell growth by 50 % (IC50) and at fixed multiples (2 and 3 times) as well as fractions (0.75, 0.5, and 0.25) of the IC50 dose. The drugs were also combined in these same dose fixed ratios and the results analyzed by the Chou and Talalay method (9). Several different schedules of combined drug exposure were utilized since the timing of drug exposure in combination may influence activity.

Results of the Combination Studies: Bryostatin 1

Initial studies included evaluation of the growth inhibitory effects of bryostatin 1, paclitaxel, and the combination of both in the MCF 7 and MDA MB 468 breast cancer cell lines, *in vitro*. Schedules examined included 30 minute pre-treatment with bryostatin 1 followed by 24 hour treatment with paclitaxel, 24 hour concomitant treatment with both paclitaxel and bryostatin 1, and 24 hour pre-treatment with bryostatin 1 followed by paclitaxel for 24 hours. Bryostatin alone was utilized at concentrations ranging from 10^{-9}M to 3 X 10^{-7}M. In combination with paclitaxel, bryostatin 1 was tested at three concentrations, 1, 10, and 100 nM. Paclitaxel was utilized at a concentration range of 10^{-10}M to 3X 10^{-8}M. Bryostatin 1 alone at an exposure time of 30 minutes or 24 hours did not result in any significant growth inhibition at any of the tested concentrations. Paclitaxel actively growth inhibited both cell lines with IC_{50}'s in the nanomolar range. Since these concentrations of bryostatin 1 were not growth inhibitory, synergy of the combination could be defined as any significant decrease in the IC_{50} compared with that of paclitaxel alone. These combinations of bryostatin 1 and paclitaxel did not demonstrate any significant change in the IC_{50} and therefore did not demonstrate synergy.

To determine if bryostatin 1 and paclitaxel would demonstrate synergy in other breast cancer cell lines. Bryostatin 1 at a concentration of 10 nM was utilized in combination with paclitaxel in MDA MB 435 and Hs578t breast cancer cell lines. The cells were exposed to 24 hours of bryostatin 1 then washed and either DMSO as vehicle control, bryostatin 1, or paclitaxel was added for an additional 24 hours. Again, no synergy was seen when bryostatin 1 was combined with paclitaxel.

To extend the previous studies, additional schedules were evaluated in the MCF 7 and MDA MB 468 cell lines. Bryostatin again at concentrations of 1, 10, and 100 nM were combined with paclitaxel at the concentrations described above. Paclitaxel and bryostatin were evaluated at three different treatment schedules; 1. Concomitant exposure for 120 hours. 2. Bryostatin 1 alone for 24 hours then the addition of paclitaxel and bryostatin 1 for 96 hours. 3. Paclitaxel alone for 24 hours then bryostatin 1 alone for 96 hours. Cell growth inhibition by these treatments was assessed on day 5. Again, no synergy was observed in either cell line under any of the experimental conditions examined.

To further assess whether bryostatin 1 was a promising agent for combination therapy in breast cancer, several additional cytotoxic chemotherapeutic agents were evaluated. These agents included vinorelbine, doxorubicin, cisplatin, and 5-fluorouracil. These agents all have known activity in the treatment of breast cancer and produce growth inhibition *in vitro* of the breast cancer cell lines utilized in these studies. In addition, they differ in their mechanisms of action when compared with eachother as well as paclitaxel. For these studies the two breast cancer cell lines, MCF 7 and MDA MB 468 were utilized. Again, three different treatment schedules were utilized, concomitant therapy for 120 hours, bryostatin 1 alone for 24 hours then in combination with drug for 96 hours, and drug alone for 24 hours then bryostatin 1 alone for 96 hours. Bryostatin 1 was added at 1, 10 or 100 nM and the cytotoxic drugs were added at a concentration range, which produced from 0 to greater than 80% growth inhibition. No synergistic combinations were identified with any drug and bryostatin 1 in either cell line.

Background: Polyamine Analogs

Polyamines are essential in both eucaryotic and procaryotic cells for growth and differentiation (10-12). It has been noted that the polyamine pathway is upregulated in tumor tissue (11). The polyamine pathway is therefore a rational target for anti-neoplastic therapy (10). Polyamine analogs are structural analogs of the endogenous polyamines. The polyamine analogs can function similarly to endogenous polyamines in terms of cell uptake, and regulation of polyamine biosynthesis and metabolism but cannot replace the polyamines' essential role in cell growth and differentiation (13-15). Several polyamine analogs have been evaluated in our laboratory, and have been shown to inhibit the growth of breast cancer cell lines as well as induce programmed cell death (16,17). Inhibition of the polyamine pathway has also been shown to modulate the activity of chemotherapeutic agents (18,19).

Results: Combination Studies with Polyamine Analogs

Evaluation of a different class of agent, i.e. polyamine analogs, in combination with chemotherapy in breast cancer cell lines *in vitro* was evaluated. Initial studies were done in the MCF 7 and MDA MB 468 breast cancer cell lines. The polyamine analogs CPENSpm and CHENSpm were utilized in combination with several chemotherapeutic agents. Treatment schedules evaluated including 120 hour concomitant, polyamine analog alone for 24 hours then

analog and drug for 96 hours, and drug alone for 24 hours followed by polyamine analog alone for 96 hours. The chemotherapeutic agents evaluated included doxorubicin, cisplatin, 5-fluorouracil, vinorelbine, paclitaxel, and docetaxel. The polyamine agents alone produce cell growth inhibition and therefore synergy in the combinations was determined by the combination index method by Chou and Talalay (9).

The results of these experiments are depicted in tables 1 through 4. The first two tables depict the results in the MCF 7 cell lines. Table one shows the results with CPENSpm with all six drugs at the three schedules examined. The treatment schedule of drug initially for 24 hours followed by CPENSpm for 96 hours demonstrates synergy at fractional growth inhibitions of greater than 50% for all six drugs evaluated. In contrast, with CHENSpm (table 2) only 5-fluorouracil, vinorelbine and paclitaxel demonstrate synergy. Again the treatment schedule of drug prior to polyamine analog is superior. For the MDA MB 468 cell line (tables 3 and 4), CPENSpm only produces synergy (at fractional growth inhibition of greater than 50%) with vinorelbine. Again, synergy is only observed when the drug precedes the analog. CHENSpm in the MDA MB 468 cell line is shown in table 4. Synergy again is only observed when the drug precedes the analog and only with 5-fluorouracil (for fractional growth inhibition of greater than 50%).

Conclusions

The combination of bryostatin 1 and paclitaxel utilizing multiple drug treatment schedules and three different concentrations of bryostatin 1 did not show any synergy in the four different breast cancer cell lines evaluated. In addition, evaluation of four other chemotherapeutic agents (with known activity in breast cancer) in combination with bryostatin 1 did not yield any synergistic combinations. Although I cannot rule out the possibility of synergy of bryostatin 1 and paclitaxel in other breast cancer models or with other drugs, a systematic and rather exhaustive evaluation of combination treatment with bryostatin 1 and paclitaxel (as well as four other chemotherapeutic agents) in several breast cancer cell lines *in vitro* makes it seem unlikely that further study of these in our models would prove fruitful. Also in light of these negative data, no experiments were performed to examine PKC activity/translocation or programmed cell death as originally proposed.

Instead, I concentrated on evaluating the therapeutic potential of another class of novel agents, polyamine analogs, in combination with chemotherapeutic agents in breast cancer cell lines, *in vitro*. In contrast to the studies initially performed with bryostatin 1, several combinations (with polyamine analogs and chemotherapeutic agents) demonstrate synergy in the MCF 7 and MDA MB 468 cell lines. Scheduling of drug exposures appears critical for synergy with drug preceding analog appearing to be the schedule required to mediate a synergistic response. Additional studies utilizing these combinations in additional cell lines, T47d, MDA MB 231, and Hs578t, are currently underway. These experiments are utilizing both CPENSpm and CHENspm in combination with the chemotherapeutic agents described above but only one schedule is being evaluated, drug alone for 24 hours followed by polyamine analog alone Additional studies underway are the evaluation of possible mechanisms underlying the synergy seen when 5 fluorouracil and polyamine analogs are combined. These studies illustrate the potential of polyamine analogs in combination therapy in the treatment of breast cancer and may aid in the rational design of combination therapy of polyamine analogs with chemotherapeutic agents in the treatment of breast cancer.

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Table 1

Treatment Schedule

Concomitant (Day 0 x 120 hr)	*Fractional Growth Inhibition*		
	0.5	*0.75*	*0.90*
Doxorubicin	antagonism	additive	additive
Cisplatin	antagonism	**synergy**	**synergy**
5 Fluorouracil	**synergy**	**synergy**	antagonism
Vinorelbine	**synergy**	**synergy**	**synergy**
Paclitaxel	antagonism	antagonism	antagonism
Docetaxel	**synergy**	additive	antagonism

Drug then CPENspm (day 0 x 24 hr then day1 x 96 hr)	*Fractional Growth Inhibition*		
	0.5	*0.75*	*0.90*
Doxorubicin	**synergy**	**synergy**	**synergy**
Cisplatin	**synergy**	**synergy**	**synergy**
5 Fluorouracil	**synergy**	**synergy**	**synergy**
Vinorelbine	**synergy**	**synergy**	**synergy**
Paclitaxel	**synergy**	**synergy**	**synergy**
Docetaxel	**synergy**	**synergy**	**synergy**

CPENspm then Drug & CPENspm (day 0 x 24 hr then day1 x 96 hr)	*Fractional Growth Inhibition*		
	0.5	*0.75*	*0.90*
Doxorubicin	antagonism	**synergy**	**synergy**
Cisplatin	antagonism	**synergy**	**synergy**
5 Fluorouracil	additive	**synergy**	**synergy**
Vinorelbine	**synergy**	**synergy**	antagonism
Paclitaxel	additive	**synergy**	**synergy**
Docetaxel	**synergy**	**synergy**	**synergy**

Evaluation of synergy, additivity, or antagonism using Combination Index analysis in the MCF 7 Cells treated with CPENSpm and chemotherapy.

Table 2

Treatment Schedule

Concomitant (Day 0 x 120 hr)	*Fractional Growth Inhibition*		
	0.5	*0.75*	*0.90*
Doxorubicin	antagonism	antagonism	antagonism
Cisplatin	antagonism	antagonism	antagonism
5 Fluorouracil	antagonism	antagonism	additive
Vinorelbine	antagonism	additive	**synergy**
Paclitaxel	antagonism	antagonism	antagonism
Docetaxel	antagonism	antagonism	antagonism

Drug then CHENspm (day 0 x 24 hr then day1 x 96 hr)	*Fractional Growth Inhibition*		
	0.5	*0.75*	*0.90*
Doxorubicin	antagonism	antagonism	antagonism
Cisplatin	antagonism	antagonism	**synergy**
5 Fluorouracil	additive	**synergy**	**synergy**
Vinorelbine	**synergy**	**synergy**	**synergy**
Paclitaxel	antagonism	**synergy**	**synergy**
Docetaxel	antagonism	antagonism	antagonism

CHENspm then Drug & CHENspm (day 0 x 24 hr then day1 x 96 hr)	*Fractional Growth Inhibition*		
	0.5	*0.75*	*0.90*
Doxorubicin	antagonism	antagonism	antagonism
Cisplatin	antagonism	antagonism	additive
5 Fluorouracil	antagonism	antagonism	antagonism
Vinorelbine	antagonism	antagonism	antagonism
Paclitaxel	antagonism	antagonism	antagonism
Docetaxel	antagonism	antagonism	antagonism

Evaluation of synergy, additivity, or antagonism using combination index analysis in the MCF 7 cell line treated with CHENspm and chemotherapy.

Table 3

Treatment Schedule

Concomitant (Day 0 x 120 hr)	*Fractional Growth Inhibition*		
	0.5	*0.75*	*0.90*
Doxorubicin	antagonism	antagonism	antagonism
Cisplatin	**synergy**	antagonism	antagonism
5 Fluorouracil	antagonism	antagonism	antagonism
Vinorelbine	antagonism	antagonism	antagonism
Paclitaxel	antagonism	antagonism	antagonism
Docetaxel	antagonism	antagonism	antagonism

Drug then CPENspm (day 0 x 24 hr then day1 x 96 hr)	*Fractional Growth Inhibition*		
	0.5	*0.75*	*0.90*
Doxorubicin	antagonism	antagonism	antagonism
Cisplatin	antagonism	antagonism	antagonism
5 Fluorouracil	**synergy**	antagonism	antagonism
Vinorelbine	**synergy**	**synergy**	**synergy**
Paclitaxel	**synergy**	additive	antagonism
Docetaxel	antagonism	antagonism	antagonism

CPENspm then Drug & CPENspm (day 0 x 24 hr then day1 x 96 hr)	*Fractional Growth Inhibition*		
	0.5	*0.75*	*0.90*
Doxorubicin	antagonism	antagonism	antagonism
Cisplatin	antagonism	antagonism	antagonism
5 Fluorouracil	antagonism	antagonism	antagonism
Vinorelbine	additive	antagonism	antagonism
Paclitaxel	antagonism	antagonism	antagonism
Docetaxel	antagonism	antagonism	antagonism

Evaluation of synergy, additivity, or antagonism using combination index analysis in the MDA MB 468 cell line treated with CPENSpm and chemotherapy.

Table 4

Treatment Schedule

Concomitant (Day 0 x 120 hr)	*Fractional Growth Inhibition*		
	0.5	*0.75*	*0.90*
Doxorubicin	antagonism	antagonism	antagonism
Cisplatin	antagonism	antagonism	antagonism
5 Fluorouracil	antagonism	antagonism	antagonism
Vinorelbine	additive	additive	additive
Paclitaxel	antagonism	antagonism	antagonism
Docetaxel	antagonism	antagonism	antagonism

Drug then CHENspm (day 0 x 24 hr then day1 x 96 hr)	*Fractional Growth Inhibition*		
	0.5	*0.75*	*0.90*
Doxorubicin	antagonism	antagonism	antagonism
Cisplatin	antagonism	antagonism	antagonism
5 Fluorouracil	antagonism	**synergy**	**synergy**
Vinorelbine	antagonism	additive	**synergy**
Paclitaxel	antagonism	antagonism	antagonism
Docetaxel	antagonism	antagonism	antagonism

CHENspm then Drug & CHENspm (day 0 x 24 hr then day1 x 96 hr)	*Fractional Growth Inhibition*		
	0.5	*0.75*	*0.90*
Doxorubicin	antagonism	antagonism	antagonism
Cisplatin	antagonism	antagonism	antagonism
5 Fluorouracil	antagonism	antagonism	additive
Vinorelbine	antagonism	antagonism	additive
Paclitaxel	antagonism	antagonism	antagonism
Docetaxel	additive	additive	additive

Evaluation of synergy, additivity, or antagonism using combination index analysis in the MDA MB 468 cell line treated with CHENSpm and chemotherapy.